

OCCURRENCE OF POTENTIALLY HAZARDOUS FUNGI IN EXPOSED BREWERY SPENT SORGHUM GRAINS

Isaac A. Onyimba, PhD

Chike I. C. Ogbonna, PhD

University of Jos, Nigeria

Chukwu, O. O. Chukwu, PhD

National Veterinary Research Institute, Vom, Nigeria

Collins C. E. Odu

University of Maiduguri, Nigeria

Abstract

Samples of environmentally exposed spent sorghum grains (SSG), commonly used as feedstuff in Nigeria, were obtained from the Jos International Brewery and screened for potentially hazardous fungi associated with them. A total of three samples, one per week, were used in the study. Truly colonizing fungi of the SSG were further determined by assessing the abilities of the fungal isolates to grow on specially formulated spent sorghum grain agar (SSGA). Fourteen fungal species belonging to nine genera were isolated from the SSG. Frequently occurring fungi included *Aspergillus niger* (89%), *A. fumigatus* (56%), *A. flavus* (78%), *Rhizopus oryzae* (78%) and *R. stolonifer* (56%). The genus *Aspergillus* had the highest number of species (28.6%) among the isolates. The true fungal colonizers of SSG were found to include *A. flavus*, *A. fumigatus*, *A. niger*, *A. ochraceus*, *Curvularia lunata*, *Geotrichum candidum*, *Humicola grisea*, *Penicillium* sp, *R. oryzae*, *R. stolonifer* and *Trichoderma harzianum*. Among the fungal isolates were species that have been known to contain either pathogenic or toxigenic strains. The findings from the experiments showed that exposed SSG can be colonized by potentially hazardous fungi and as such, caution should be exercised in its use as a feedstuff.

Keywords: Brewery, fungi, hazardous, sorghum, spent grains

Introduction:

Brewery spent grain (BSG), one of the major by-products of beer production, has been a feed resource for animal production in Nigeria. The

grains provide protein, fibre and energy, and can be used in a variety of diets (Westendorf & Wohlt, 2002; Szponar et al., 2003). The presence of cellulose, hemicellulose and lignin, and also the amount of readily available substances such as sugars and amino acids aid in its utilization as feed for ruminants (Bisaria et al., 1997).

High moisture content of BSG (80 to 85%) together with polysaccharide and protein content makes it particularly susceptible to microbial growth and subsequent deterioration in a short period of time (7 to 10 days) (Stojceska et al., 2008). This can constrain its appeal as an industrial feedstock.

In the use of agricultural industrial by-products such as BSG in Nigeria, nutrient content has been the farmer's basic concern. The possible presence of pathogenic and toxigenic microorganisms in the feedstuffs is often overlooked. Gill and Best (1998) listed animal feed as one of the sources of microorganisms to animals. Essential as nutrient content may be, sanitary quality of feeds remains a major consideration if healthy animals are going to be produced. Ingestion of feed materials containing hazardous microorganisms or metabolites may lead to disease conditions and possible mortality of animals. Humans who come into contact with such infected animals may also be affected. Studies elsewhere have associated some animal feeds with toxigenic strains of fungi of public health concern (Bilgram et al., 1995; White & Torman, 1995).

Considering the health hazard posed by contaminated feed materials, and also the attendant socioeconomic impact, it is needful to undertake this study. This research was therefore designed to investigate the occurrence of potentially hazardous fungi in exposed brewery spent sorghum grains.

Materials and Methods:

Collection and Processing of Experimental Samples

The spent sorghum grain (SSG) samples used in the work were obtained from the Jos International Brewery. The samples were collected from the floor of the spent grain collection point where the spent grains had been spread for sun-drying. A total of three samples were collected, one sample per week. The samples were aseptically collected into sterile polythene bags using sterile gloves and immediately transported to the laboratory where they were kept in a refrigerator at 4 °C.

Isolation of Fungi from the Experimental Samples

Fungi were isolated from the experimental samples using the plating method described by Warcup (1950). A weighed spent sorghum grain (SSG) sample (0.1g) was placed in sterile Petri-dishes. A volume of 15ml of freshly prepared Malt Extract Agar (MEA) medium was cooled to 44 °C and then

poured into each of the plates. Three drops of chloramphenicol (0.1g/l) was added to the MEA plates to suppress bacteria. The plates, with their contents, were then swirled for adequate mixing and then allowed to solidify. A total of 27 MEA plates were employed for the isolation of fungi from the three SSG samples collected. Nine (9) MEA plates were thus used per sample. Nine (9) MEA plates were incubated at 25 °C for the isolation of mesophilic microorganisms. A second set of 9 MEA plates were incubated at 37 °C for the isolation of thermotolerant microorganisms while the last set of 9 MEA plates were incubated at 45 °C for the isolation of thermophilic microorganisms. Control plates (which contained culture media without SSG) were incubated at each of the isolation temperatures for comparative purposes. The incubated plates were examined after 4-14 days for presence of fungal species. Subcultures were made to obtain pure cultures. The percentage frequencies of occurrence of the isolates were determined using the method employed by Alli et al. (1998).

Identification of Isolates

The isolates were first differentiated based on cultural characteristics including colony morphology (appearance of the colonies on solid media). The characteristics observed at this stage included shape colour, size, texture, elevation and outline of the colony. There was need to grow some of the isolates on specific media for better observation of some characteristics. *Aspergillus* and *Penicillium* species were identified after cultivation on Czapeck Agar at 25 °C. This was to enable easy separation of individual species through colour difference of their colonies (Samson et al, 1984).

After studying the characteristics of the isolates on solid media, they were then examined under the microscope. The microscopy of the fungal isolates included direct examination of fungal plates with their mycelial structures undisturbed and also the examination of slide preparations of the isolates. The nature of hyphae and fruiting structures were useful in the identification of the fungi. The 100x oil immersion objective was used for observation of details of spore attachment, surface texture, ornamentation of hyphae and spores. After the process of characterization, the fungal isolates were identified by making references to identification manuals including, Barnett and Hunter (1972), Raper and Fenell (1977), Pitt (1979), Domsch et al. (1980), Von Arx (1981), Samson et al. (1984), and Pitt and Hockings (1997). Reference was also made to existing stock cultures of some of these species of organisms in the Department of Plant Science and Technology of the University of Jos.

Determination of True Fungal Colonisers of the Spent Sorghum Grains

The fungal isolates were plated out on specially formulated Spent Sorghum Grain Agar (SSGA). The SSGA was prepared by adding two percent of spent grain powder to a basal medium containing (0.5g); Potassium Dihydrogen Phosphate (1.0g); Potassium Chloride (0.5g); Magnesium Sulphate (0.2g); Calcium Chloride (0.1g); Agar (20.0g) and Distilled Water (1000ml). The spent grain was the sole source of carbon and nitrogen. The medium was sterilized by autoclaving for 15 minutes at 121°C. It was allowed to cool and was then dispensed into sterile Petri dishes. Thereafter, it was allowed to set. Each previously isolated fungus was cultured on an SSGA plate and incubated at the same temperature at which it was isolated for 4-14 days. The ability of a fungus to grow on the SSGA showed it to be a real colonizer of the substrate and not just a non colonizing contaminant.

Statistical analyses of results were carried out where necessary using Analysis of Variance (Zar, 1974).

Results:

Fourteen fungal species belonging to nine genera were isolated from the spent sorghum grain (SSG). The fungal isolates were made up of twelve species of mesophilic fungi (86%), one thermotolerant species (7%) and one thermophilic species (7%). Temperature had a significant difference ($P < 0.05$) on the number of fungal isolates.

The genus *Aspergillus* had the highest number of species. The most frequently occurring fungi were *Aspergillus niger* (89%), *A. flavus* (78%), *A. fumigatus* (56%), *Rhizopus oryzae* (78%) and *R. stolonifer* (56%). The least frequently isolated fungi were *H. fresenii* (11%) and *H. grisea* (11%) followed by *Penicillium* sp (22%) and *T. harzianum* (22%). *H. fresenii* was isolated in week 1 only while *H. grisea* and *T. harzianum* were isolated in week 3 alone. There were no significant differences ($P > 0.05$) in the numbers of isolates from the different weeks. Details of the fungal isolates and their percentage frequencies of occurrence are given in Table 1.

Table 1: Fungal Isolates from Weekly Samples of Spent Sorghum Grains and their Percentage Frequencies of Occurrence

FUNGI	SAMPLE 1			SAMPLE 2			SAMPLE 3			TOTAL	OCCURRENCE FREQUENCY(%)
	*P1	P2	P3	P4	P5	P6	P7	P8	P9		
<i>Aspergillus flavus</i>	+	+	+	+	-	+	+	+	-	7	78
<i>A. fumigatus</i>	-	+	-	+	+	-	+	+	-	5	56
<i>A. niger</i>	+	-	+	+	+	+	+	+	+	8	89
<i>A. ochraceous</i>	+	-	+	+	-	-	+	-	-	4	44
<i>Chrysionilla sitophila</i>	-	+	-	+	-	+	-	+	-	4	44
<i>Curvularia lunata</i>	-	-	-	+	+	-	-	+	+	4	44
<i>Geotrichum candidum</i>	-	+	-	+	-	-	+	-	-	3	33
<i>Helicostylum fresenii</i>	-	-	+	-	-	-	-	-	-	1	11
<i>Humicola grisea</i>	-	-	-	-	-	-	-	+	-	1	11
<i>H. insolens</i>	+	-	-	+	-	-	-	-	+	3	33
<i>Penicillium</i> sp	-	+	-	-	+	-	-	-	-	2	22
<i>Rhizopus oryzae</i>	+	-	+	+	+	+	+	-	+	7	78
<i>R. stolonifer</i>	-	+	+	-	+	+	-	-	+	5	56
<i>Trichoderma harzianum</i>	-	-	-	-	-	-	+	+	-	2	22
TOTAL	7	5	5	8	6	6	7	6	6	56	

*P = Plate

Keys: + = fungus present, - = fungi absent, S = sample

The true fungal colonizers of SSG among the isolates included *A. flavus*, *A. fumigatus*, *A. niger*, *A. ochraceous*, *Curvularia lunata*, *Geotrichum candidum*, *H. grisea*, *Penicillium* sp, *R. oryzae*, *R. stolonifer* and *T. harzianum*. These species were able to grow on the spent sorghum grain agar (SSGA).

Discussion:

The predominance of mesophiles among the microbial isolates was due to the cold weather condition of Jos North, the study area. The importance of environmental factors to microbial activity has been stressed by Ayerst (1969), Pitt (1975) and Brown (1976).

The isolation of fungal species belonging to the genera *Aspergillus*, *Penicillium* and *Rhizopus*, among others, is in conformity with the findings of Sodhi et al (1985) who isolated eight fungal species of the genera *Aspergillus*, *Fusarium*, *Mucor*, *Penicillium* and *Rhizopus* from brewery spent grains and that of Monica et al. (2007) who reported the isolation of fungal

species belonging to the genera *Aspergillus*, *Fusarium*, *Mucor*, *Penicillium*, *Rhizopus* and *Cladosporium*.

The predominance of *Aspergillus* species (28.6%) in the present study conforms with the findings of Monica et al. (2007) and Gerbaldo et al. (2011) who reported similar predominance of *Aspergillus* species in brewery spent grains. In the findings of the first authors, *Aspergillus* species made up 42% of the total number of isolates and was followed by *Rhizopus* species (32.5%). The same trend was observed in the current study. This high frequency of occurrence of *Aspergillus* species could be an indication of the abilities of members of the genus to produce the necessary enzymes for the utilization of the nutritional components of the SSG. *Aspergillus* species have been reported to be more prevalent in tropical soils (Waksman, 1939) including the Nigerian soil (Ogbonna, 1980).. The abundance of the *Aspergilli* in the environment plus their high reproductive rate and resistant characteristic (Pitt & Hockings, 1997) may have given them a competitive advantage over the other isolated species. The partial differences observed between the mycoflora of the present study and that of some other workers may be attributed to differences in processing and type of grains used. While barley spent grains were used in the works cited, sorghum spent grains were used in the present study. These factors would naturally affect the nutritional composition of the brewer's grains and ultimately affect their colonization patterns.

The growth of the true fungal colonizers on the spent sorghum grains agar was due to their ability to utilize the spent grains as sole source of carbon and nitrogen. These fungi have been isolated from various ecological environments, plant and food sources (Domsch et al.1980; Samson et al., 1984; Pitt & Hockings, 1997). The fungal isolates that were not able to grow on the chemically defined agar media could have been surface contaminants.

Among the isolates were species that are known to contain either pathogenic or toxigenic strains. Species of the genus *Aspergillus* are the causative agents of aspergillosis in animals and man. *A. fumigatus*, one of the isolates, is the primary causative agent of aspergillosis (Bennet, 1979). Another isolate, *A. flavus*, under favourable conditions, have been shown to produce the hepatocarcinogens, aflatoxin B1, B2, and sterigmatocystin (D'Mello, 2004). Aflatoxins are toxic to most experimental and domesticated animals and man. *A. ochraceous* produces Ochratoxin A, a hepatotoxin which has been shown to be toxic to ducklings, mice and rats (Davis & Diener, 1987). The predominance of *Aspergillus* species and their high frequencies of occurrence pose health risks to animals feeding on such contaminated spent grains. Though *A. niger* had the highest occurrence frequency (89%), it may not constitute a health threat as the fungus is usually regarded as benign, and has been widely used in food processing. There have

been few reports, however, of mycotoxin production by *A. niger* (Abarca et al 1994). Other highly occurring fungi that were able to colonize the spent grains included *R. oryzae* (78%) and *R. stolonifer* (56%). In the findings of Rabie et al. (1985), *R. stolonifer* was moderately toxic to ducklings, while maize meal on which *R. oryzae* had been grown was toxic to ducklings and rats. The isolation of a *Penicillium* species is noteworthy considering that the genus *Penicillium* is considered to be of great importance to humans and domesticated animals with respect to natural poisoning outbreaks (Davis & Diener, 1987).

Conclusion:

The results of the isolation experiment have shown that exposed brewery spent sorghum grains can be colonized by potentially-hazardous fungi. Caution should therefore be exercised in the use of such grains as feedstuff. Farmers feeding their animals with brewery spent grains which often come in a wet form should use the feed material as soon as it is collected from the brewery. Where the spent grains cannot be used immediately, they should be dried quickly to prevent the growth of hazardous microorganisms. The potentially hazardous fungi, particularly those with high frequencies of occurrence, isolated in this study will further be screened for actual mycotoxin production.

References:

- Abarca, M. L., Bragulat, M. R., Castella, G. and Cabanes, F. G. (1994). Ochratoxin A production by strains of *Aspergillus niger* var. *niger*. *Applied Environmental Microbiology*, 60, 2650-2652.
- Alli, A. I., Ogbonna, C. I. C. & Rahman, A. T. M. F. (1998). Hydrolysis of certain Nigerian cereal starches using fungal amylase. *Nigerian Journal of Biotechnology*, 9(1), 24-36.
- Ayerst, G. (1969). The effects of moisture and temperature on growth and spore germination in some fungi. *Journal of Stored Products Research*, 5, 127-141.
- Barnett, H. L. & Hunter, B. B. (1972). *Illustrated genera of imperfect fungi* (3rd ed.). Minneapolis: Burgess Publishing Company.
- Bennet, J. E. (1979). *Aspergillus* species. In G. Mandell, R. G. Douglas & J. E. Bennet (Eds.), *Principles and practices of infectious diseases* (pp. 2002-2008). New York: John Wiley and Sons.
- Bilgram, K.S., Sinha, K. K., Ansaria, A. A. and Rahman, M. (1995). Widespread occurrence of toxigenic *Alternaria* in cereals and oil seeds. *Indian Phytopathology*, 48 (2), 150-153.

- Bisaria, R., Madan, M. and Vasudevan, P. (1997). Utilization of agro-residues as animal feed through bioconversion. *Bioresource Technology*, 59, 5-8.
- Brown, M. E. (1957). *The physiology of fishes*. New York: Academic Press.
- Davis, N. D. & Dienner, U. I. (1987). Mycotoxins. In L. R. Beuchat (Ed.), *Food and beverage mycology* (pp. 517-570). New York: Van Nostrand Reinhold.
- D'Mello, J.P.F. (2004). Contaminants and Toxins in Animal Feeds. Retrieved from <http://www.fao.org/agrippa/publications/ToC3.htm>
- Domsch, K. H., Gam, W. & Anderson, T. (1980). *Compendium of soil fungi*. London: Academic Press Limited.
- Gerbaldo, G. A., Pereyra, C. M., Cavaglieri, L. R., Ruiz, F., Pascual, L., Dalcero, A. M. and Barberis, I. L. (2011). Surveillance of aflatoxin and microbiota related to brewer's grain destined for swine feed in Argentina. Retrieved from <http://www.hindawi.com/journals/vmi/2011/912480/>
- Gill, C. and Best, R. (1998). Antibiotic resistance in USA: scientists to look more closely. *Feed International*, 19(8), 16-17.
- Monica, M.S., Simas, M. B., Botura, B. C., Sabino, M., Mallmann, C.A., Bitencourt, T. C., Batatinha, M. J. (2007). Determination of fungal microbiota and mycotoxins in brewersgrain used in dairy cattle feeding in the State of Bahia, Brazil. *Food Control*, 18(5), 404-408.
- Ogbonna. C. I. C. (1980). The effects of Nigerian Fungi on Nigerian Wood. (Unpublished doctoral thesis). University of Technology Aston, Birmingham.
- Pitt, J. I. (1975). Food spoilage by xerophilic fungi. In R. B. Duckworth (Ed.), *Water relations of foods* (pp. 304-391). London: Academic Press.
- Pitt, J. I. (1979). *The genus Penicillium and its teleomorphic states*. London: Academic Press.
- Pitt, J. I. & Hockings, A. D. (1997). *Fungi and food spoilage* (2nd ed.). London: Blackie Academic and Professional.
- Rabie, C. J., Lubben, A., Schipper, M. A. A., van Herden, F. R. and Fincham, J. E. (1985). Toxigenicity of *Rhizopus* species. *International Journal of Food Microbiology*, 1, 263-270.
- Raper, K. B. and Fennel, D. I. (1977). *The genus Aspergillus*. Baltimore: Williams and Wilkins Co.
- Samson, R.A., Hoekstra, E.S. and Van Oorschot, C.A.N. (1984). *Introduction to food-borne fungi* (2nd ed). Netherlands: Institute of the Royal Netherlands Academy of Arts and Sciences.
- Stojceska, V., Ainsworth, P., Plunkett, A. and Ibanoglu, S. (2008). The recycling of brewery processing by-product into ready- to-eat snacks using extrusion technology. *Jornal of Cereal Science*, 47, 469-479.

- Szponar, B., Pawlik, K. J., Gamian, A. and Dey, E.S. (2003). Protein fraction of barley spent grain as a new simple medium for growth and sporulation of soil actinobacteria. *Biotechnology Letters*, 25, 1717-1721.
- Von Arx, J. A. (1981). *The genera of fungi sporulating in pure culture*, (3rd ed). Verlag. Germany: Vaduz, J. Cramer.
- Waksman, S. A., Umbreit, W. W. and Cordon, T. C. (1939). Thermophilic actinomycetes and fungi in soils and composts. *Soil Science*, 47, 37-67.
- Warcup, J. H. (1950). The soil plate method for isolation of fungi from the soil. *Nature*, 166, 177-178.
- Westendorf, M.L. and Wohlt, J.E. (2002). Brewing by-products: their use as animal feeds. *Veterinary Clinics of North America: Food Animal Practice*, 18(2), 233-252.
- White, D. G. and J. Torman (1995). Post harvest application of low rates of DMX – 7 mold inhibitor on storage fungi of corn during ambient air drying and storage. *Applied Engineering in Agriculture*, 11(4), 573 – 576.
- Zar, J. H. (1974). *Biostatistical Analysis*. Englewood Cliffs, New Jersey: Prentice Hall.